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# Ankyrin–Tiam1 Interaction Promotes Rac1 Signaling and Metastatic Breast Tumor Cell Invasion and Migration

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**Abstract.** Tiam1 (T-lymphoma invasion and metastasis 1) is one of the known guanine nucleotide (GDP/GTP) exchange factors (GEFs) for Rho GTPases (e.g., Rac1) and is expressed in breast tumor cells (e.g., SP-1 cell line). Immunoprecipitation and immunoblot analyses indicate that Tiam1 and the cytoskeletal protein, ankyrin, are physically associated as a complex in vivo. In particular, the ankyrin repeat domain (ARD) of ankyrin is responsible for Tiam1 binding. Biochemical studies and deletion mutation analyses indicate that the 11-amino acid sequence between amino acids 717 and 727 of Tiam1 (<sup>717</sup>GEGTDVAVKRS<sup>727</sup>L) is the ankyrin-binding domain. Most importantly, ankyrin binding to Tiam1 activates GDP/GTP exchange on Rho GTPases (e.g., Rac1).

Using an *Escherichia coli*-derived calmodulin-binding peptide (CBP)-tagged recombinant Tiam1 (amino acids 393–728) fragment that contains the ankyrin-binding domain, we have detected a specific binding interaction between the Tiam1 (amino acids 393–738)

fragment and ankyrin in vitro. This Tiam1 fragment also acts as a potent competitive inhibitor for Tiam1 binding to ankyrin. Transfection of SP-1 cell with Tiam1 cDNAs stimulates all of the following: (1) Tiam1–ankyrin association in the membrane projection; (2) Rac1 activation; and (3) breast tumor cell invasion and migration. Cotransfection of SP1 cells with green fluorescent protein (GFP)-tagged Tiam1 fragment cDNA and Tiam1 cDNA effectively blocks Tiam1–ankyrin colocalization in the cell membrane, and inhibits GDP/GTP exchange on Rac1 by ankyrin-associated Tiam1 and tumor-specific phenotypes. These findings suggest that ankyrin–Tiam1 interaction plays a pivotal role in regulating Rac1 signaling and cytoskeleton function required for oncogenic signaling and metastatic breast tumor cell progression.

**Key words:** Tiam1 • ankyrin • Rac1 signaling • invasion/migration • metastatic breast tumor cells

## Introduction

Members of the Rho subclass of the ras superfamily (small molecular masses GTPases, e.g., Rac1, RhoA, and Cdc42) are known to be associated with changes in the membrane-linked cytoskeleton (Ridley and Hall, 1992; Hall, 1998). For example, activation of Rac1, RhoA, and Cdc42 has been shown to produce specific structural changes in the plasma membrane cytoskeleton associated with membrane ruffling, lamellipodia, filopodia, and stress fiber formation (Ridley and Hall, 1992; Hall, 1998). The coordinated activation of these GTPases is thought to be a possible mechanism underlying cell motility, an obvious prerequisite for metastasis (Jiang et al., 1994; Dickson and Lippman, 1995; Lauffenburger and Horwitz, 1996).

Several guanine nucleotide exchange factors (GEFs,<sup>1</sup> the dbl or DH family) have been identified as oncogenes because of their ability to upregulate Rho GTPase activity during malignant transformation (Van Aelst and D'Souza-Schorey, 1997). One of these GEFs is Tiam1 (T-lymphoma invasion and metastasis 1), which was identified by retroviral insertional mutagenesis and selected for its invasive cell behavior in vitro (Habets et al., 1994, 1995). This molecule is largely hydrophilic and contains several functional domains found in signal transduction proteins. For example, the COOH-terminal region of the Tiam1 molecule has a Dbl homology (DH) domain (Hart et al., 1991, 1994; Habets et al., 1994) and an adjacent pleckstrin homology

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<sup>1</sup>Abbreviations used in this paper: ARD, ankyrin repeat domain; CBP, calmodulin-binding peptide; DH, Dbl homology; GFP, green fluorescent protein; GFP-SBD, GFP-tagged spectrin binding domain; GEF, guanine nucleotide exchange factor; HA, hemagglutinin; PH, pleckstrin homology; PHn, NH<sub>2</sub>-terminal PH; Rh, rhodamine; S1P, sphingosine-1-phosphate; SBD, spectrin binding domain; Tiam1, T lymphoma invasion and metastasis 1.

(PH) domain, which exists in most GEFs (Hart et al., 1991, 1994; Habets et al., 1994; Lemmon et al., 1996). In particular, the DH domain of these proteins exhibits GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (Hart et al., 1991, 1994). Tiam1 also contains an additional PH domain, a Disc-large homology region (DHR; Habets et al., 1994; Pontings and Phillips, 1995), and a potential myristoylation site in the NH<sub>2</sub>-terminal part of the protein (Habets et al., 1994).

Overexpression of both NH<sub>2</sub> and COOH terminally truncated as well as full-length Tiam1 proteins induces the invasive phenotype in otherwise noninvasive lymphoma cell lines (Michiels et al., 1995). It is also well established that Tiam1 is capable of activating Rac1 in vitro as a GEF, and induces membrane cytoskeleton-mediated cell shape changes, cell adhesion, and cell motility (Woods and Bryant 1991; Michiels et al., 1995; Nobes and Hall, 1995; Van Leeuwen et al., 1995). These findings have prompted investigations into the mechanisms involved in the regulation of Tiam1. In fact, it has been found that addition of certain serum-derived lipids (e.g., sphingosine-1-phosphate [S1P] and LPA) to T-lymphoma cells promotes Tiam1-mediated Rac1 signaling and T-lymphoma cell invasion (Stam et al., 1998). A Tiam1 transcript has been detected in breast cancer cells (Habets et al., 1995). Tiam1 is shown to function as a GEF in activating Rac1 signaling in breast tumor cells (Bourguignon et al., 2000). The question of how this molecule is regulated in invasive and metastatic processes of breast cancer cells is addressed in the present study.

Ankyrin belongs to a family of cytoskeletal proteins that mediate linkage of integral membrane proteins with the spectrin-based skeleton in regulating a variety of biological activities (Bennett, 1992; Bennett and Gilligan, 1993; De Matteis and Morrow, 1998). Presently, at least three ankyrin genes have been identified: *ankyrin 1* (ANK1 or *ankyrin R*), *ankyrin 2* (ANK2 or *ankyrin B*), and *ankyrin 3* (ANK3 or *ankyrin G*) (Lambert et al., 1990; Lux et al., 1990; Tse et al., 1991; Otto et al., 1991; Peters and Lux 1993; Kordeli et al., 1995; Peters et al., 1995). All ankyrin species (e.g., ANK1, ANK2, and ANK3) are monomers comprised of two highly conserved domains and a variable domain. Both conserved domains are located in the NH<sub>2</sub>-terminal region and include a membrane-binding site (~89–95 kD, also called the ankyrin repeat domain [ARD]; Davis and Bennet, 1990; Lux et al., 1990), and a spectrin binding domain (SBD, ~62 kD; Platt et al., 1993). The striking feature shared by all three forms of ankyrins is the repeated 33-amino acid motif present in 24 contiguous copies within the ARD. The ARD of ANK1, ANK2, and ANK3 is highly conserved. A number of tumor cells express ankyrin such as ANK1 and ANK3 (Bourguignon et al., 1998a,b; Zhu and Bourguignon, 2000). Most recently, we have found that ankyrin's ARD interacts with the adhesion molecule, CD44, and promotes tumor cell migration (Zhu and Bourguignon, 2000). In addition, the ARD domain (also referred to as cdc 10 repeats, cdc10/SW16 repeats, and SW16/ANK repeats) has been detected in a number of functionally distinct proteins participating in protein-protein binding and protein-DNA interactions (Davis and Bennett, 1990; Lux et al., 1990).

In this study, we have focused on the regulatory aspect of Tiam1-Rac1 signaling in metastatic breast tumor cells (SP-1 cell line). Our results indicate that Tiam1 interacts with ankyrin in vivo and in vitro. In particular, the ankyrin repeat domain (ARD) is directly involved in Tiam1 binding. Biochemical analyses show that the Tiam1 fragment (amino acids 393–738) contains an ankyrin-binding site and competes for Tiam1 binding to ankyrin. Most importantly, the binding of ankyrin, in particular, the ankyrin repeat domain (ARD), to Tiam1 activates Rho-like GTPases such as Rac1. Overexpression of Tiam1 in SP-1 cells by transfecting Tiam1 cDNA induces Tiam1-ankyrin association in the cell membrane, Rac1 signaling, and metastatic phenotypes. Both Tiam1-ankyrin interaction and tumor-specific behaviors are significantly inhibited by cotransfecting SP-1 cells with the Tiam1 (amino acids 393–738) fragment cDNA and Tiam1 cDNA. Our observations suggest that Tiam1 interaction with ankyrin promotes Rho GTPase activation and cytoskeletal changes required for metastatic breast tumor cell invasion and migration.

## Materials and Methods

### Cell Culture

Mouse breast tumor cells (e.g., SP1 cell line; provided by Dr. Bruce Elliott, Department of Pathology and Biochemistry, Queen's University, Kingston, Ontario, Canada) were used in this study. Specifically, the SP1 cell line was derived from a spontaneous intraductal mammary adenocarcinoma that arose in a retired female CBA/J breeder in the Queen's University animal colony. These cells were capable of inducing lung metastases by sequential passage of SP1 cells into mammary gland (Elliott et al., 1988). These cells were cultured in RPMI 1640 medium supplemented with either 5 or 20% FCS, folic acid (290 mg/l), and sodium pyruvate (100 mg/l). COS-7 cells were obtained from American Type Culture Collection and grown routinely in DME containing 10% FBS, 1% glutamine, 1% penicillin, and 1% streptomycin.

### Antibodies and Reagents

For the preparation of polyclonal rabbit anti-Tiam1 antibody, specific synthetic peptides (~15–17 amino acids unique for the COOH-terminal sequence of Tiam1) were prepared by the Peptide Laboratories of the Department of Biochemistry and Molecular Biology using an automatic synthesizer (model ACT350; Advanced Chemtech). These Tiam1-related polypeptides were conjugated to polylysine and subsequently injected into rabbits to raise the antibodies. The anti-Tiam1-specific antibody was collected from each bleed and stored at 4°C containing 0.1% azide. The anti-Tiam1 IgG fraction was prepared by conventional DEAE-cellulose chromatography. Mouse monoclonal anti-hemagglutinin (HA epitope) antibody (clone 12 CA5) and mouse monoclonal anti-green fluorescent protein (GFP) antibody were purchased from Boehringer Mannheim and PharMingen, respectively. *Escherichia coli* (*E. coli*)-derived GST-tagged Rac1/Cdc42 and GST-tagged RhoA was provided by Dr. Richard A. Cerione (Cornell University, Ithaca, NY) and Dr. Martin Schwartz (Scripps Research Institute, La Jolla, CA), respectively. Mouse monoclonal erythrocyte ankyrin (ANK1) and ANK3 antibodies were prepared as described previously (Bourguignon et al., 1993a). Rabbit anti-ANK3 antibody was provided by Dr. L.L. Peters (Jackson Laboratory, Bar Harbor, ME; Peters et al., 1995).

### Cloning, Expression, and Purification of GST-tagged Ankyrin Repeat Domain (GST-ARD) and GFP-tagged Spectrin Binding Domain (GFP-SBD) of Ankyrin

pGEX-2TK recombinant plasmid expressing GST-ARD (NH<sub>2</sub>-terminal portion of ankyrin, residues 1–834) was constructed as follows. Two pGEX-2TK recombinant plasmids pA3-79 (expressing epithelial Ank3 NH<sub>2</sub>-terminal 1–455 amino acids) and pA3-88 (expressing epithelial Ank3

NH<sub>2</sub>-terminal 317–834 amino acids; Peters et al., 1995) were provided by Dr. L.L. Peters from the Jackson Laboratory. The two plasmids were digested by EcoRI (one of pGEX-2TK vector cloning sites) and NheI (in ankyrin cDNA 1,176 bp) sequentially. The digested products were run in 1% agarose gel and purified with a purification kit (QIAGEN). The larger cDNA fragment in pA3-79-digested products (containing the pGEX-2TK vector and ankyrin cDNA 1–1,176 bp) and the smaller one in pA3-88-digested products (containing ankyrin cDNA 1,176–2,556 bp) were cut and purified. These two cDNA fragments were ligated and transformed to INVαF'-competent cells. The obtained clones were sequenced to verify the correct generation of the full-length ARD.

Spectrin binding domain (SBD) cDNA of human erythrocyte ankyrin was cloned into the eukaryotic expression vector, GFPN1 (CLONTECH Laboratories, Inc.) using the PCR-based cloning strategy. Ankyrin's SBD cDNA was amplified by PCR with two specific primers (left, 5'-CGCTC-GAGATGAAGGCTGAGAGCGGGGATTCC-3' and right, 5'-ATAA-GCTTCAGGGGCGTCGGGGTCTTCT-3') linked with specific enzyme digestion site (XhoI and HindIII). The PCR product, which was digested with XhoI and HindIII, was purified with QIAquick PCR purification kit (QIAGEN). Ankyrin's SBD cDNA fragment was cloned into GFPN1 vector digested with XhoI and HindIII. The cDNA sequence was confirmed by nucleotide sequencing analysis. The GFP-tagged spectrin binding domain (GFP-SBD) of ankyrin is expressed as an 89-kD polypeptide in SP1 or COS-7 cells by SDS-PAGE and immunoblot analyses. The 89-kD GFP-SBD (but not ARD) displays specific spectrin binding property as described previously (Platt et al., 1993). Subsequently, GFP-SBD was isolated from anti-GFP-conjugated affinity columns and used in various in vitro binding experiments as described below.

## Expression Constructs

Both the full-length mouse Tiam1 cDNA (FL1591) and the NH<sub>2</sub> terminally truncated Tiam1 cDNA (C1199) were provided by Dr. John G. Colard (The Netherlands Cancer Institute, The Netherlands). Specifically, the full-length Tiam1 (FL1591) cDNA was cloned into the eukaryotic expression vector, pMT2SM. The truncated C1199 Tiam1 cDNA (carrying a hemagglutinin epitope [HA] tag at the 3' end) was cloned in the eukaryotic expression vector, pUTSV1 (Eurogentec, Belgium).

The deletion construct, HA-tagged C1199 Tiam1Δ717-727 (deleting the sequence between amino acids 717 and 727 of Tiam1) was derived from C1199 Tiam1 using QuickChange™ site-directed mutagenesis kit (Stratagene). In brief, two complementary mutagenic oligonucleotide primers containing the desired deletion (5'-CCCAACCATCAACCAGGTGTT-TGAGGGAATATTTGATG-3') was designed and synthesized. First, the cycling reaction, using 30-ng double-stranded DNA template of C1199 Tiam1 plasmid and two complementary primers, was performed to produce mutated cDNA according to the manufacturer's instruction. Subsequently, 1 μl of the DpnI restriction enzyme (10 U/μl) was added directly to the cycling reaction products to digest the parental supercoiled double-stranded DNA. This DpnI-treated cDNA was used to transform supercompetent cells (e.g., *Episcurian coli* XL 1-blue). Finally, the deletion construct was confirmed by DNA sequencing.

The Tiam1 (amino acids 393–728) fragment was cloned into calmodulin-binding peptide (CBP)-tagged vector (pCAL-n; Stratagene) using the PCR-based cloning strategy. Using human Tiam1 cDNA as a template, the Tiam1 fragment was amplified by PCR with two specific primers (left, 5'-AACTCGAGATGAGTACCAACAGTGAG-3' and right, 5'-AAAAAGCTTTCAGCCATCTGGAACAGTGTATC-3') linked with a specific enzyme digestion site (XhoI or HindIII). The PCR product, which was digested with XhoI and HindIII, was purified with QIAquick PCR purification kit (QIAGEN). The Tiam1 fragment cDNA was cloned into pCAL-n vector digested with XhoI and HindIII. The inserted Tiam1 fragment sequence was confirmed by nucleotide sequencing analyses. The recombinant plasmids were transformed to BL21-DE3 to produce CBP-tagged Tiam1 fragment fusion protein. This fusion protein was purified from bacteria lysate by calmodulin affinity resin column (Sigma Chemical Co.).

The Tiam1 fragment cDNA was also cloned into pEGFPN1 vector (CLONTECH Laboratories, Inc.) digested with XhoI and HindIII to create GFP-tagged Tiam1 fragment cDNA. The inserted Tiam1 fragment sequence was confirmed by nucleotide sequencing analyses. This GFP-tagged Tiam1 fragment cDNA was used for transient expression in SP1 cells as described below. The GFP-tagged Tiam1 fragment is expressed as a 68-kD polypeptide in SP1 or COS-7 cells by SDS-PAGE and immunoblot analyses.

## Cell Transfection

To establish a transient expression system, cells (e.g., SP-1 or COS-7 cells) were transfected with various plasmid DNAs including Tiam1 cDNAs (e.g., the full-length mouse Tiam1 cDNA [FL1591], or HA-tagged C1199 Tiam1 cDNA, or HA-tagged C1199 Tiam1Δ717-727 cDNA, or GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA (cotransfection), or vector control constructs) using electroporation methods. In brief, cells (e.g., SP-1 or COS-7 cells) were plated at a density of 10<sup>6</sup> cells per 100-mm dish, and were transfected with 25 μg/dish plasmid DNA using electroporation at 230 V and 960 μFD with a gene pulser (Bio-Rad). Transfected cells were grown in 5 or 20% FCS-containing culture medium for at least 24–48 h. Various transfectants were analyzed for the expression of Tiam1 or HA-tagged (or GFP-tagged) Tiam1 mutant proteins by immunoblot, immunoprecipitation, and functional assays as described below.

## Immunoprecipitation and Immunoblotting Techniques

SP-1 cells or COS cells (e.g., untransfected or transfected by various Tiam1 cDNAs including the full-length mouse Tiam1 cDNA [FL1591] or HA-tagged C1199 Tiam1 cDNA) were first extracted with a solution containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% NP-40 buffer, followed by solubilizing in SDS sample buffer, and analyzed by SDS-PAGE (with 7.5% gel). Separated polypeptides were transferred onto nitrocellulose filters. After blocking nonspecific sites with 3% BSA, the nitrocellulose filters were incubated with 5 μg/ml either of rabbit anti-Tiam1 or mouse anti-HA (or preimmune serum) plus peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:10,000 dilution), respectively. In controls, peroxidase-conjugated normal mouse IgG or preimmune rabbit IgG was also incubated with anti-Tiam1-mediated immunocomplex. The blots were developed using ECL chemiluminescence reagent (Amersham Life Science) according to the manufacturer's instructions.

In some cases, SP-1 cells (transfected with HA-tagged C1199 Tiam1 cDNA, or HA-tagged C1199 Tiam1Δ717-727 cDNA, or GFP-tagged Tiam1 fragment cDNA, or cotransfected with HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA) were immunoblotted with anti-HA antibody (5 μg/ml) or anti-GFP antibody (5 μg/ml), respectively, followed by incubation with HRP-conjugated goat anti-mouse IgG (1:10,000 dilution) at room temperature for 1 h.

SP-1 cells were also immunoprecipitated with rabbit anti-Tiam1 (5 μg/ml) or mouse anti-ankyrin antibodies (e.g., 5 μg/ml of either mouse anti-ANK3 antibody or mouse anti-ANK1 antibody), followed by immunoblotting/reblotting with ankyrin antibodies (e.g., 1 μg/ml mouse anti-ANK3 antibody, or 5 μg/ml mouse anti-ANK1 antibody, or 1 μg/ml rabbit anti-Tiam1), respectively, followed by incubation with HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:10,000 dilution) at room temperature for 1 h. In reblotting controls, both peroxidase-conjugated normal mouse IgG or rabbit preimmune IgG was also used. The blots were developed using ECL chemiluminescence reagent (Amersham Life Science) according to the manufacturer's instructions.

## Effects of Synthetic Peptides on Ankyrin-Tiam1 Interaction

Nitrocellulose discs (1-cm diam) were coated with ~1 μg of a panel of synthetic peptides including the ankyrin-binding region peptide (<sup>717</sup>GEGTDAVKRS<sup>727</sup>L), a scrambled peptide (GRATLEGSDKV) and another Tiam1-related peptide (<sup>399</sup>GTIKRAPFLG<sup>409</sup>P; synthesized by Dr. Eric Smith, University of Miami). After coating, the unoccupied sites on the discs were blocked by incubation with a solution containing 20 mM Tris-HCl, pH 7.4, and 0.3% BSA at 4°C for 2 h. The discs were incubated with various concentration of <sup>125</sup>I-labeled cytoskeletal proteins (erythrocyte ankyrin/ARD/ankyrin's SBD/spectrin; ~3000 cpm/ng) at 4°C for 2 h in 1 ml binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% BSA).

In some experiments, <sup>125</sup>I-labeled Tiam1 (~3,000 cpm/ng) was incubated with ankyrin-coated beads in the presence of various concentrations (10<sup>-10</sup>–10<sup>-6</sup> M) of unlabeled synthetic peptide (e.g., <sup>717</sup>GEGTDAVKRS<sup>727</sup>L or the scrambled sequence, GRATLEGSDKV, or another Tiam1-related peptide, <sup>399</sup>GTIKRAPFLG<sup>409</sup>P) at 4°C for 2 h in 1 ml binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% BSA). <sup>125</sup>I-labeled Tiam1 fragment (~3,000 cpm/ng) was also incubated with beads containing 1.0 μg of each of the following four proteins: intact ankyrin, ARD, or spectrin binding domain of ankyrin (GFP-SBD), or GFP alone.

After binding, the peptide-coated discs (or cytoskeletal protein-conjugated beads) were washed three times in the binding buffer, and the radioactivity associated with the peptide-coated discs (or cytoskeletal protein-conjugated beads) was estimated. As a control, the ligands were also incubated with uncoated nitrocellulose discs (or beads) to determine the binding observed because of the stickiness of various ligands. Nonspecific binding was observed in these controls. In the peptide competition assay, the specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used.

### **Binding of Ankyrin or ARD to Tiam1 In Vitro**

Aliquots (0.5–1.0  $\mu$ g of protein) of purified Tiam1 (e.g., intact Tiam1, or C1199 Tiam1, or Tiam1 fragment)-conjugated beads were incubated in 0.5 ml of binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100) containing various concentrations (10–800 ng/ml) of  $^{125}$ I-labeled intact ankyrin (purified from human erythrocytes; 5,000 cpm/ng protein) or  $^{125}$ I-labeled recombinant ARD fragment at 4°C for 4 h. Specifically, equilibrium binding conditions were determined by performing a time course (1–10 h) of  $^{125}$ I-labeled ankyrin (or ARD) binding to Tiam1 at 4°C. The binding equilibrium was found to be established when the in vitro ankyrin (or ARD)-Tiam1 binding assay was conducted at 4°C after 4 h. After binding, beads were washed extensively in binding buffer, and the bead-bound radioactivity was counted.

As a control,  $^{125}$ I-labeled ankyrin or  $^{125}$ I-labeled ARD was also incubated with uncoated beads to determine the binding observed because of the nonspecific binding of various ligands. Nonspecific binding, which represented ~20% of the total binding, was always subtracted from the total binding. Our binding data are highly reproducible. The values expressed in the Results represent an average of triplicate determinations of three to five experiments with an SD less than  $\pm$  5%. In some cases,  $^{125}$ I-ankyrin (1–10 ng) was incubated with a polyacrylamide gel containing purified Tiam1 (obtained from anti-Tiam1 affinity column chromatography) in the absence or the presence of 100-fold excess amount of unlabeled ankyrin/spectrin (in the same binding buffer as described above) for 1 h at room temperature. After incubation, the gel was washed five times with the same binding solution and analyzed by autoradiographic analyses.

An in vitro binding assay designed to measure the stoichiometry of GST-ARD fusion protein and C1199 Tiam1 was also carried out. Specifically, in each reaction, 15–60  $\mu$ l of glutathione-Sepharose bead slurry containing GST-ARD or GST alone was suspended in 0.5 ml of binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100). Purified C1199 Tiam1 (0.5–1.0  $\mu$ g) was added to the bead suspension in the absence or the presence of an excess amount of CBP-tagged Tiam1 fragment (100  $\mu$ g) at 4°C for 4 h. After binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using 150  $\mu$ l of 50 mM Tris-HCl, pH 8.0, buffer containing 30 mM glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was determined by SDS-PAGE and Coomassie blue staining followed by densitometric scanning using a software NIH Image V1.54. The amount of ARD (mol) per C1199 Tiam1 (mol) was calculated. Values represent relative binding abilities averaged from three experiments  $\pm$  SEM.

### **Binding of $^{125}$ I-Labeled Ankyrin to C1199 Tiam1 and the Mutant Protein**

SP1 cells were transfected with HA-tagged C1199 Tiam1 cDNA, or HA-tagged C1199 Tiam1 $\Delta$ 717–727 cDNA, or vector alone. These transfectants were extracted with a solution containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% NP-40, and immunoprecipitated with anti-HA immunofluorescence beads. Subsequently, aliquots (50 ng proteins) of these beads were incubated with 0.5 ml of a binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100) in presence of various concentrations (10–400 ng/ml) of  $^{125}$ I-labeled ankyrin (5,000 cpm/ng protein) at 4°C for 5 h. After binding, beads were washed extensively in binding buffer and the bead-bound radioactivity was counted.

As a control,  $^{125}$ I-labeled ankyrin was also incubated with uncoated beads to determine the binding observed because of the nonspecific binding of the ligand. Nonspecific binding, which represented ~15–20% of the total binding, was always subtracted from the total binding. The values expressed in the Results represent an average of triplicate determinations of three to five experiments with an SD less than  $\pm$  5%.

### **Tiam1-mediated GDP/GTP Exchange for Rho GTPases**

Purified *E. coli*-derived GST-tagged GTPases (e.g., Rac1, Cdc42, or RhoA; 20 pmol) were preloaded with GDP (30  $\mu$ M) in 10  $\mu$ l buffer containing 25 mM Tris-HCl, pH 8.0, 1 mM DTT, 4.7 mM EDTA, 0.16 mM  $\text{MgCl}_2$ , and 200  $\mu$ g/ml BSA at 37°C for 7 min. To terminate preloading procedures, additional  $\text{MgCl}_2$  was added to the solution (reaching a final concentration of 9.16 mM) as described previously (Zhang et al., 1995). Tiam1 was isolated from COS-7 cells (transfected with either the full-length Tiam1 cDNA or HA-tagged C1199 Tiam1 cDNA) or SP1 cells (transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1 cDNA, GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA [as cotransfection] or vector alone) using anti-Tiam1 (or anti-HA or anti-GFP)-conjugated beads. In some cases, ankyrin-associated Tiam1 was isolated from SP1 cells (transfected with HA-tagged C1199 Tiam1 cDNA, GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA [as a cotransfection], or vector alone) using anti-ankyrin-conjugated beads.

Subsequently, 2 pmol of Tiam1, isolated from untransfected or transfected cells according to the procedures described above, was preincubated with no ankyrin or ankyrin (e.g., 1  $\mu$ g/ml of either intact ankyrin or ARD), followed by adding to the reaction buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , 100  $\mu$ M AMP-PNP, 0.5 mg/ml BSA, and 2.5  $\mu$ M GTP- $\gamma$ - $^{35}$ S (~1,250 Ci/mmol). Subsequently, 2.5 pmol GDP-loaded GST-tagged Rho GTPases (e.g., Rac1, RhoA, or Cdc42) or GDP-treated GST were mixed with the reaction buffer containing Tiam1 and GTP- $\gamma$ - $^{35}$ S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM  $\text{MgCl}_2$  as described previously (Michiels et al., 1995; Zhang et al., 1995). The termination reactions were filtered immediately through nitrocellulose filters, followed by one wash with the termination buffer. The filters were dissolved completely in scintillation fluid, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTP- $\gamma$ - $^{35}$ S bound to Tiam1 or control sample (pre-immune serum-conjugated Sepharose beads) in the absence of Rho GTPases (e.g., Rac1, Cdc42, or RhoA) was subtracted from the original values. Data represent an average of triplicates from three to five experiments. SD < 5%.

### **Double Immunofluorescence Staining**

SP1 cells (untransfected or transfected with various plasmid DNAs such as HA-tagged C1199 Tiam1 cDNA, GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA [as a cotransfection], or vector alone) were first washed with PBS (0.1 M phosphate buffer, pH 7.5, and 150 mM NaCl) buffer and fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment followed by staining with different immunoreagents. Specifically, untransfected cells were incubated with rhodamine (Rh)-conjugated mouse anti-ANK3 (50  $\mu$ g/ml) and fluorescein (FITC)-conjugated rabbit anti-Tiam1 (50  $\mu$ g/ml), respectively. HA-tagged C1199 cDNA-transfected cells were stained with Rh-conjugated mouse anti-ANK3 antibody (50  $\mu$ g/ml) and FITC-conjugated mouse anti-HA IgG (50  $\mu$ g/ml), respectively. GFP-tagged Tiam1 fragment cDNA-transfected cells were labeled with Rh-conjugated anti-ANK3 (50  $\mu$ g/ml). Some SP1 transfectants (cotransfected with Tiam1 fragment cDNA and HA-tagged C1199 Tiam1 cDNA) were stained with Rh-conjugated anti-HA (50  $\mu$ g/ml) or Rh-conjugated anti-ANK3 (50  $\mu$ g/ml), respectively. To detect nonspecific antibody binding, vector-transfected cells were labeled with Rh-conjugated anti-ANK3 (50  $\mu$ g/ml) followed by incubating with FITC-conjugated anti-HA (50  $\mu$ g/ml). No anti-HA labeling was observed in such control samples. In some experiments, GFP-tagged Tiam1 fragment cDNA-transfected cells were also incubated with Rh-labeled rabbit pre-immune IgG (50  $\mu$ g/ml). No nonspecific rhodamine staining was detected in these samples. The FITC- and Rh-labeled samples were examined with a confocal laser scanning microscope (MultiProbe 2001 inverted CLSM system; Molecular Dynamics).

### **Tumor Cell Migration and Invasion Assays**

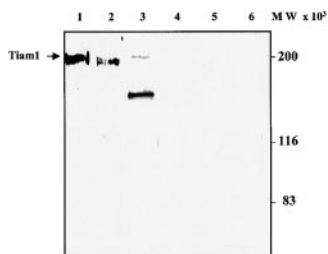
24 transwell units were used for monitoring in vitro cell migration and invasion as described previously (Merzak et al., 1994; Bourguignon et al., 1998b, 2000). Specifically, the 5- $\mu$ m porosity polycarbonate filters coated with the reconstituted basement membrane substance Matrigel (Collabo-

rative Research) were used for the cell invasion assay (Merzak et al., 1994; Bourguignon et al., 1998b). The 5- $\mu$ m porosity polycarbonate filters (without Matrigel coating) were used for the cell migration assay (Merzak et al., 1994; Bourguignon et al., 1998b, 2000). SP-1 cells transfected with various Tiam1-related cDNAs (e.g., full-length Tiam1 cDNA, HA-tagged C1199 Tiam1 cDNA, or GFP-tagged Tiam1 fragment cDNA, or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA [cotransfection], or vector alone) ( $\sim 10^4$  cells/well in PBS, pH 7.2, untreated or treated with cytochalasin D [20  $\mu$ g/ml] or DMSO alone) were placed in the upper chamber of the transwell unit. The growth medium containing high glucose DME supplemented by 10% FBS was placed in the lower chamber of the transwell unit. After an 18-h incubation at 37°C in a humidified 95% air/5% CO<sub>2</sub> atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration and invasion processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (Merzak et al., 1994; Zhu and Bourguignon, 2000). Each assay was set up in triplicate and repeated at least five times. All data were analyzed statistically by *t* test and statistical significance was set at *P* < 0.01.

## Results

### Identification of the GEF, Tiam1 in Breast Tumor Cells (SP-1 Cells)

Rho GTPases such as Rac1 become activated when bound GDP is exchanged for GTP by a process catalyzed by GEFs such as Tiam1 (Habets et al., 1994). A Tiam1 transcript has been detected previously in breast cancer cells (Habets et al., 1995). In this study, we have analyzed Tiam1 expression (at the protein level) in SP-1 breast tumor cells. Immunoblot analysis, using anti-Tiam1 antibody designed to recognize the specific epitope located at the COOH terminus of Tiam1 molecule, reveals a single polypeptide ( $\sim 200$  kD; Fig. 1, lane 1). This 200-kD Tiam1-like molecule, expressed in SP-1 cells, is very similar to the Tiam1 detected in COS-7 cells that were transiently transfected with the full-length Tiam1 cDNA (Fig. 1, lane 2) or NH<sub>2</sub> terminally truncated C1199 Tiam1 cDNA (Fig. 1, lane 3 revealing primarily C1199 Tiam1 [160 kD] and a low level of endogenous Tiam1 [200 kD]). We believe that the Tiam1 detected in SP-1 cells or COS-7 transfectants, revealed by anti-Tiam1-mediated immunoblot, is specific

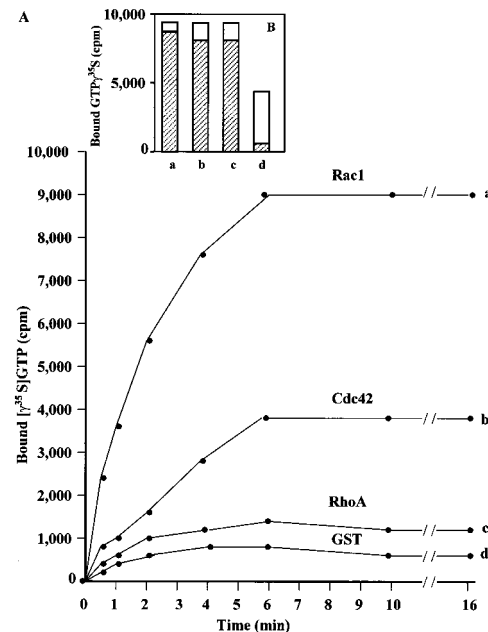


**Figure 1.** Detection of Tiam1 expression in SP-1 cells or COS-7 transfectants. SP-1 and COS-7 cells, which were transfected with the full-length Tiam1 cDNA (FL1591) or NH<sub>2</sub> terminally truncated C1199 Tiam1 cDNA or vector alone, were solubilized in SDS sample

buffer and analyzed by SDS-PAGE and immunoblot as described in Materials and Methods. (lane 1) Anti-Tiam1-mediated immunoblot of SP-1 cells. (lane 2) Anti-Tiam1-mediated immunoblot of COS-7 cells transfected with the full-length Tiam1 cDNA (FL1591). (lane 3) Anti-Tiam1-mediated immunoblot of COS-7 cells transfected with the NH<sub>2</sub> terminally truncated C1199 Tiam1 cDNA. (lane 4) Immunoblot of SP-1 cells with preimmune rabbit serum. (lane 5) Immunoblot of COS-7 cells, which were transfected with Tiam1 cDNA [FL1591], with preimmune rabbit serum. (lane 6) Immunoblot of COS-7 cells, which were transfected with the NH<sub>2</sub> terminally truncated C1199 Tiam1 cDNA, with preimmune rabbit serum.

since no protein is detected in these cells using preimmune rabbit IgG (Fig. 1, lanes 4–6).

To confirm that the Tiam1-like molecule functions as a GDP/GTP exchange factor (or a GDP-dissociation stimulator protein) for Rac1, we have isolated Tiam1 from SP-1 cells using anti-Tiam1-conjugated Sepharose beads. Our results indicate that SP1's Tiam1 activates GDP/GTP exchange on GST-Rac1 (Fig. 2 A, a) and, to a lesser extent, on GST-Cdc42 (Fig. 2 A, b) and GST-RhoA (Fig. 2 A, c). The initial onset of the exchange reaction on GST-Rac1



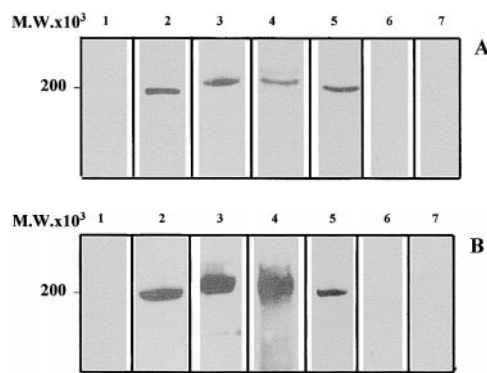
**Figure 2.** Tiam1-mediated GDP/GTP exchange for Rho GTPases. Purified *E. coli*-derived GST-tagged GTPases (e.g., Rac1, Cdc42, or RhoA) were preloaded with GDP. First, 2 pmol Tiam1 that was isolated from SP1 cells or COS-7 transfectants was added to the reaction buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 100  $\mu$ M AMP-PNP, 0.5 mg/ml BSA, and 2.5  $\mu$ M GTP- $\gamma$ -<sup>35</sup>S ( $\sim 1,250$  Ci/mmol). Subsequently, 2.5 pmol GDP-loaded GST-tagged Rho GTPases (e.g., Rac1, RhoA, Cdc42, or GST alone) were mixed with the reaction buffer containing Tiam1 and GTP- $\gamma$ -<sup>35</sup>S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer as described in Materials and Methods. The termination reactions were filtered immediately through nitrocellulose filters, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTP- $\gamma$ -<sup>35</sup>S bound to Tiam1 or control sample (preimmune serum-conjugated Sepharose beads) in the absence of Rho GTPases (e.g., Rac1, Cdc42, or RhoA) was subtracted from the original values. Data represent an average of triplicates from three to five experiments. SD < 5%. (A) Kinetics of GTP- $\gamma$ -<sup>35</sup>S bound to GDP-loaded GST-Rac1 (a), GST-Cdc42 (b), or GST-RhoA (c), or GST alone (d) in the presence of Tiam1 (isolated from SP-1 cells). (B) The maximal level of GTP- $\gamma$ -<sup>35</sup>S bound to GST-Rac1 in the presence of Tiam1 isolated from SP1 grown in 5% FCS (a, shaded bar) or 20% FCS (a, blank bar); or the full-length Tiam1 (1,591) isolated from COS-7 transfectants grown in 5% FCS (b, shaded bar) or 20% FCS (b, blank bar); or the C1199 Tiam1 isolated from COS-7 transfectants grown in 5% FCS (c, shaded bar) or 20% FCS (c, blank bar); or Tiam1 isolated from vector-transfected COS-7 cells grown in 5% FCS (d, shaded bar) or 20% FCS (d, blank bar).

occurs within 0.5–1 min after the addition of Tiam1, and the reaction reaches its maximal level  $\sim 16$  min after Tiam1 addition (Fig. 2 A, a). In contrast, the initial rate of Tiam1-catalyzed GDP/GTP exchange on Cdc42 (Fig. 2 A, b) and RhoA (Fig. 2 A, c) appears to be significantly lower than that detected on Rac1 (Fig. 2 A, a). In the control samples, the amount of [ $^{35}$ S]GTP- $\gamma$ -S associated with GST alone is found to be significantly decreased (Fig. 2 A, d). Further analysis indicates that the ability of Tiam1 isolated from SP-1 cells to promote GDP/GTP exchange on Rac1 (Fig. 2 B, a) is identical to that carried out by the Tiam1 isolated from COS-7 transfected with the full-length Tiam1 cDNA (Fig. 2 B, b) or NH<sub>2</sub> terminally truncated C1199 Tiam1 cDNA (Fig. 2 B, c). Therefore, we believe that the Tiam1 in SP-1 cells clearly functions as a GDP/GTP exchange factor for Rho-like GTPases such as Rac1 GTPase.

We have also noticed that Tiam1 isolated from nontransfected COS-7 cells grown in the presence of 20% FCS is capable of catalyzing GDP/GTP exchange on Rac1 at a much higher level (Fig. 2 B, d, blank bar) than Tiam1 isolated from nontransfected COS-7 cells grown in the presence of 5% FCS (Fig. 2 B, d, shaded bar). This observation is consistent with the previous findings that some serum components play an important role in upregulating the ability of Tiam1 to promote GDP/GTP exchange on Rac1 (Stam et al., 1998). In SP1 cells (Fig. 2 B, a, blank and shaded bars) or Tiam1 cDNA-transfected COS-7 cells (Fig. 2 B, b and c, blank and shaded bars), neither high nor low serum causes significant changes in the ability of Tiam1 to catalyze GDP/GTP exchange on Rac1. These differential serum effects on the activity of Tiam1 isolated from low or high Tiam1-expressing cells await future investigation.

### Interaction between Tiam1 and the Cytoskeletal Proteins, Ankyrin

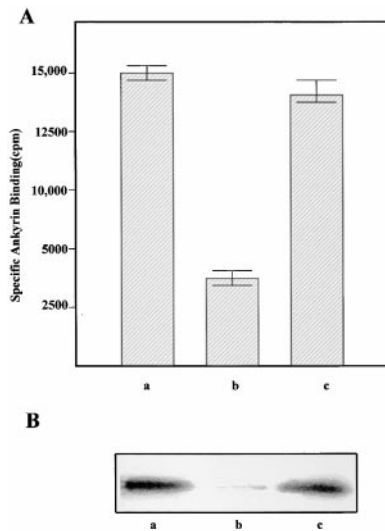
Certain cytoskeleton proteins, such as ankyrin, are known to be involved in regulating a variety of cellular activities (Bennett, 1992; Bennett and Gilligan, 1993; Bourguignon, 1996; Bourguignon et al., 1998a; De Matteis and Morrow, 1998). Both ankyrin1 (ANK1) and ankyrin 3 (ANK3) have been shown to be expressed in breast tumor cells (Bourguignon et al., 1998b, 1999). In this study, we have carried out anti-ANK1 or anti-ANK3-mediated immunoprecipitation of SP-1 cellular proteins, followed by anti-Tiam1 immunoblot (Fig. 3, A and B, lane 2) and anti-ANK1 (Fig. 3 A, lane 3)/ANK3 (Fig. 3 B, lane 3) immunoblot, respectively. Our results indicate that the Tiam1 band is revealed in anti-ankyrin (e.g., ANK1 or ANK3)-mediated immunoprecipitated materials (Fig. 3, A and B, lane 2). Apparently, Tiam1 is coprecipitated with ANK1 and/or ANK3 (revealed by reblotting with anti-ANK1/ANK3 antibody; Fig. 3, A and B). In control samples, immunoblotting of anti-ANK1 or anti-ANK3-immunoprecipitated material using rabbit preimmune serum (Fig. 3, A and B, lane 1) does not reveal any protein associated with this material. Anti-Tiam1-mediated immunoprecipitation of SP-1 cellular proteins, followed by anti-ANK1 (Fig. 3 A, lane 4) or anti-ANK3 (Fig. 3 B, lane 4)-mediated immunoblot also shows that both ANK1 (Fig. 3 A, lane 4) and ANK3 (Fig.



**Figure 3.** Detection of Tiam1-ankyrin complex in SP1 cells. SP1 cells ( $5 \times 10^5$  cells) were solubilized by 1% NP-40 buffer and processed for anti-ankyrin or anti-Tiam1-mediated immunoprecipitation, followed by immunoblotting with anti-Tiam1 or anti-ANK1 (or anti-ANK3) antibody, respectively, as described in Materials and Methods. (A) Analysis of Tiam1-ANK1 complex (lane 1). Anti-ANK1-mediated immunoprecipitation followed by immunoblotting with rabbit preimmune serum. (lanes 2 and 3) Detection of Tiam1 in the complex by mouse anti-ANK1-mediated immunoprecipitation, followed by immunoblotting with rabbit anti-Tiam1 antibody (lane 2) or reblotting with mouse anti-ANK1 antibody (lane 3). (lanes 4–7) Detection of ANK1 in the complex by rabbit anti-Tiam1-mediated immunoprecipitation, followed by immunoblotting with mouse anti-ANK1 antibody (lane 4) or reblotting with rabbit anti-Tiam1 antibody (lane 5), or peroxidase-conjugated normal mouse IgG (lane 6) or peroxidase-conjugated rabbit preimmune IgG (lane 7). (B) Analysis of Tiam1-ANK3 complex: (lane 1) anti-ANK3-mediated immunoprecipitation followed by immunoblotting with rabbit preimmune serum. (lanes 2 and 3) Detection of Tiam1 in the complex by mouse anti-ANK3-mediated immunoprecipitation, followed by immunoblotting with rabbit anti-Tiam1 antibody (lane 2) or reblotting with mouse anti-ANK3 antibody (lane 3). (lanes 4–7) Detection of ANK3 in the complex by rabbit anti-Tiam1-mediated immunoprecipitation, followed by immunoblotting with mouse anti-ANK3 antibody (lane 4) or reblotting with rabbit anti-Tiam1 antibody (lane 5), or peroxidase-conjugated normal mouse IgG (lane 6), or peroxidase-conjugated rabbit preimmune IgG (lane 7).

3 B, lane 4) can be coprecipitated with Tiam1 (revealed by reblotting with anti-Tiam1 antibody; Fig. 3, A and B, lane 5). In controls, very little material is detected in this anti-Tiam1-mediated immunocomplex using either normal mouse IgG (Fig. 3, A and B, lane 6) or rabbit preimmune serum-mediated immunoblot (Fig. 3 A, lane 7). These findings clearly establish the fact that Tiam1 and ankyrin (e.g., ANK1 and ANK3) are closely associated with each other as an *in vivo* complex in breast tumor cells.

Further analyses using an *in vitro* binding assay show that  $^{125}$ I-labeled ankyrin (i.e., erythrocyte ankyrin [ANK1]) binds Tiam1, which was isolated from SP1 cells, specifically (Fig. 4 A, a). In addition, we have used  $^{125}$ I-labeled ankyrin to bind purified Tiam1 (isolated from SP-1 cells) on a gel (Fig. 4 B, a). Our data indicate that Tiam1 binds to ankyrin (ANK1; Fig. 4 B, a) directly. In the presence of an excess amount of unlabeled ankyrin, the binding between ankyrin and Tiam1 is greatly reduced (Fig. 4, A and B, b). Other cytoskeletal proteins, such as spectrin,

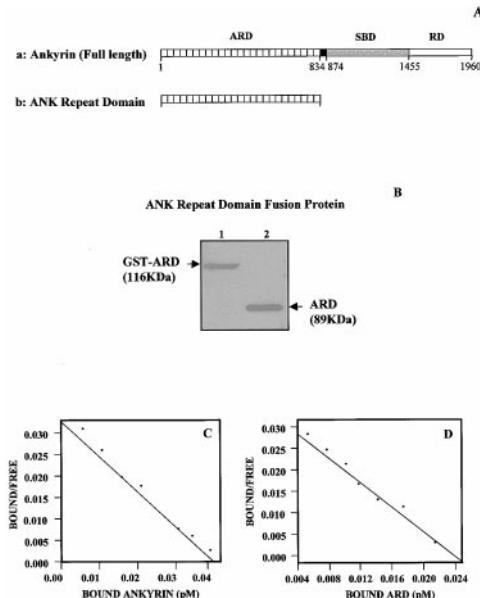


**Figure 4.** Binding interaction between Tiam1 and the cytoskeletal protein ankyrin. (A) Tiam1 (isolated from SP-1 cells) bound to the anti-Tiam1 immunobeads were incubated with  $^{125}\text{I}$ -labeled ankyrin (5,000 cpm/ng protein) in the absence (a) or the presence of 100-fold excess of unlabeled ankyrin (b) or spectrin (c). After binding, the immunobeads were washed extensively in binding buffer and the bead-bound radioactivity was estimated. (B) Autoradiogram of  $^{125}\text{I}$ -labeled ankyrin binding to a polyacrylamide gel containing purified Tiam1 (isolated from SP-1 cells) in the absence (a) or the presence of 100-fold excess of unlabeled ankyrin (b) or spectrin (c).

do not interfere with ankyrin binding to Tiam1 (Fig. 4, A and B, c). However, the precise functional domain of ankyrin involved in Tiam1 binding remains to be determined.

The  $\text{NH}_2$ -terminal region of ankyrin's membrane binding domain (Fig. 5 A, a) is comprised of a tandem array of 24 ankyrin repeats (so-called ankyrin repeat domain, ARD; Fig. 5 A, b). The question of whether the membrane-binding domain of ankyrin (in particular, ARD) is involved in Tiam1 binding is now addressed in this study. First, the pGEX-2TK recombinant plasmid encoding ARD ( $\text{NH}_2$ -terminal portion of ankyrin, from amino acids 1 to 834) was constructed with a GST tag and expressed in *E. coli* (Zhu and Bourguignon, 2000). The purified GST-tagged ARD fusion protein is expressed as a 116-kD protein (Fig. 5 B, lane 1). After the removal of GST tag by thrombin digestion, the ARD itself is found to be an 89-kD polypeptide (Fig. 5 B, lane 2), which is similar to the 89-kD ARD obtained by enzymatic digestion of erythrocyte ankyrin (Davis and Bennett, 1990).

Next, we have used the ARD fragment of ANK3 (GST-ARD) and purified Tiam1 to identify the exact Tiam1 binding site(s) on the ankyrin molecule. Specifically, we have tested the binding of Tiam1 to  $^{125}\text{I}$ -labeled intact erythrocyte ankyrin (ANK1), or  $^{125}\text{I}$ -labeled GST-ARD fragment of ANK3, under equilibrium binding conditions. Scatchard plot analyses indicate that intact erythrocyte ankyrin (ANK1) binds to Tiam1 at a single site (Fig. 5 C) with high affinity (an apparent dissociation constant [ $K_d$ ] of  $\sim 0.72$  nM). This ankyrin-Tiam1 binding interaction is



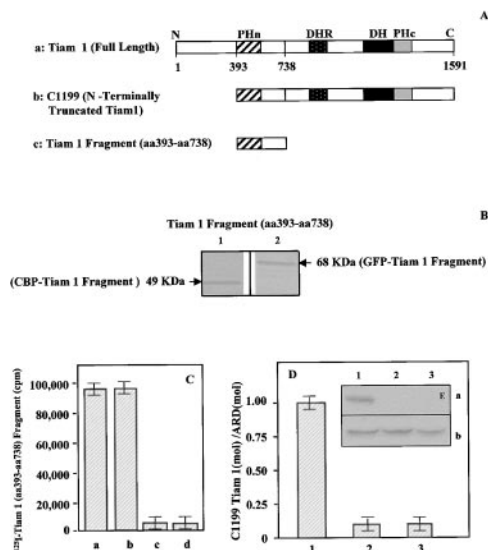
**Figure 5.** Ankyrin structure and ankyrin repeat domain (ARD) fusion protein. (A, a) Schematic illustration of functional domains in full-length ankyrin: ankyrin repeat domain (ARD), spectrin binding domain (SBD), and regulatory domain (RD). (A, b) ARD cDNA was constructed according to the strategy described in Materials and Methods. This ARD cDNA construct encodes for the  $\text{NH}_2$ -terminal region of the ankyrin membrane binding domain with a tandem array of 24 ankyrin repeats. (B) A Coomassie blue stain of the 116-kD GST-ARD fusion protein purified by affinity column chromatography (lane 1), and the 89-kD ARD (lane 2) after the removal of GST by thrombin digestion. (C and D) Scatchard plot analyses of the equilibrium binding between  $^{125}\text{I}$ -labeled ankyrin and Tiam1. Various concentrations of  $^{125}\text{I}$ -labeled ankyrin (e.g., intact erythrocyte ankyrin [ANK1] or ARD) were incubated with purified Tiam1-coupled beads at  $4^\circ\text{C}$  for 4 h. After binding, beads were washed extensively in binding buffer and the bead-bound radioactivity was counted. As a control,  $^{125}\text{I}$ -labeled ankyrin or  $^{125}\text{I}$ -labeled ARD was also incubated with uncoated beads to determine the binding observed because of the nonspecific binding of various ligands. Nonspecific binding, which represented  $\sim 20\%$  of the total binding, was always subtracted from the total binding. Our binding data are highly reproducible. Scatchard plot analysis of the equilibrium binding data between  $^{125}\text{I}$ -labeled intact erythrocyte ankyrin (ANK1) and Tiam1 (C); and Scatchard plot analysis of the equilibrium binding data between  $^{125}\text{I}$ -labeled ARD and Tiam1 (D).

comparable in affinity to Tiam1 binding ( $K_d \sim 1.42$  nM) to ANK3's ARD fragment (Fig. 5 D). These findings strongly support the notion that ankyrin (in particular, the ARD) is involved in the Tiam1 binding site.

### Determination of Tiam1's Ankyrin-binding Domain

Previous studies indicate that Tiam1's  $\text{NH}_2$ -terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain (i.e., a sequence between amino acids 393 and 738 of Tiam1; Fig. 6 A, a-c) is required for the activation of Rac1 signaling pathways leading to membrane ruffling and c-Jun  $\text{NH}_2$ -terminal kinase activation (Michiels





**Figure 6.** Properties of Tiam1 and Tiam1 mutant proteins. (A, a) The full-length Tiam1 contains DH, *dbl* homology domain; DHR, *discs-large* homology domain; two pleckstrin homology (PH) domains (including the NH<sub>2</sub>-terminal PH [PHn] and the COOH-terminal PH [PHc]). (A, b) The NH<sub>2</sub> terminally truncated C1199 Tiam1 encodes the COOH-terminal 1,199 amino acids. (A, c) The Tiam1 fragment encodes the sequence between amino acids 393 and 738. (B) Characterization of Tiam1 fragment (amino acids 393–738) fusion proteins. Coomassie blue staining of *E. coli*-derived CBP-Tiam1 fragment fusion protein purified by calmodulin affinity column chromatography (lane 1); and GFP-tagged Tiam1 fragment fusion protein purified by anti-GFP-conjugated affinity column chromatography (lane 2). (C, a) Binding of <sup>125</sup>I-Tiam1 fragment to ankyrin. (C, b) Binding of <sup>125</sup>I-Tiam1 fragment to ARD. (C, c) Binding of <sup>125</sup>I-Tiam1 fragment to the spectrin binding domain of ankyrin. (C, d) Binding of <sup>125</sup>I-Tiam1 fragment to spectrin. (D and E) Binding analysis between GST-ARD and the recombinant C1199 Tiam1 in vitro. In each reaction, glutathione-Sepharose bead slurry containing GST-ARD or GST alone was suspended in the binding buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% BSA, and 0.05% Triton X-100). Purified C1199 Tiam1 (0.5–1.0 μg) was added to the bead suspension in the absence or the presence of an excess amount of CBP-tagged Tiam1 fragment (100 μg) at 4°C for 4 h. After binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using 150 μl of 50 mM Tris-HCl, pH 8.0, buffer containing glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was determined by SDS-PAGE and Coomassie blue staining, followed by densitometric scanning using a software NIH Image V1.54. The amount of ARD (mol) per C1199 Tiam1 (mol) was calculated. Values represent relative binding abilities averaged from three experiments ± SEM. (D) The amount of C1199 Tiam1 (mol) associated with GST-ARD (mol) was measured in the absence (lane 1) or the presence of the recombinant Tiam1 fragment (lane 2) or C1199 Tiam1 associated with GST-coated beads (lane 3) using SDS-PAGE and Coomassie blue staining followed by densitometric analyses. (E) Coomassie blue staining of C1199 Tiam1 associated with GST-ARD in the absence (lane 1) or the presence of recombinant Tiam1 fragment (lane 2) or C1199 Tiam1 associated with GST-coated beads (lane 3).

et al., 1997; Stam et al., 1997). Using a 49-kD *E. coli*-derived CBP-tagged Tiam1 fragment (i.e., amino acids 393–738 of Tiam1; Fig. 6 B, lane 1) and an in vitro binding assay (Fig. 6 C), we have detected a specific binding inter-

action between the Tiam1 fragment and ankyrin (Fig. 6 C, a) and ARD (Fig. 6 C, b) but not the spectrin binding domain of ankyrin (Fig. 6 C, c) or spectrin (Fig. 6 C, d).

Furthermore, we have evaluated the binding interaction between GST-ARD fusion protein and the recombinant C1199 Tiam1 (NH<sub>2</sub> terminally truncated Tiam1; Fig. 6 D). First, glutathione-Sepharose beads containing GST-ARD were incubated with C1199 Tiam1 in the absence (Fig. 6, D and E, lane 1) or the presence of an excess amount of Tiam1 fragment (Fig. 6, D and E, lane 2). In controls, C1199 Tiam1 was also added to Sepharose beads containing GST alone (Fig. 6, D and E, lane 3). After binding, the GST fusion protein was eluted with its associated C1199 Tiam1 using a buffer containing glutathione. The amount of eluted GST fusion protein and C1199 Tiam1 was determined by SDS-PAGE and Coomassie blue staining (Fig. 6 E) followed by densitometric scanning analyses (Fig. 6 D). Our results indicate that the stoichiometry of ARD–C1199 Tiam1 interaction is ~1:1 (Fig. 6 D, lane 1, and Fig. 6 E, lane 1, a and b). In the presence of an excess amount (~100-fold) of recombinant Tiam1 fragment, the binding between ankyrin ARD and C1199 Tiam1 is significantly reduced (Fig. 6, D and E, lane 2, a and b). The control beads containing GST alone fail to bind C1199 Tiam1 (Fig. 6 D, lane 3, and Fig. 6 E, lane 3, a and b). These observations suggest that ankyrin ARD directly interacts with Tiam1, and that the ankyrin-binding domain (ARD)-containing Tiam1 fragment act as a potent competitive inhibitor of Tiam1 binding to ankyrin in vitro.

Protein sequence analyses show that Tiam1 contains the sequence <sup>717</sup>GEGTDAVKRS<sup>727</sup>L (in mouse), or <sup>717</sup>GEGTEAVKRS<sup>727</sup>L (in human) that shares a great deal of sequence homology with the ankyrin-binding domain of the cell adhesion receptor, CD44 family (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). To test whether the sequence GEGTDAVKRSL of Tiam1 protein is in fact involved in ankyrin binding, we have examined the ability of an 11-amino acid synthetic peptide, identical to GEGTDAVKRSL, to bind various cytoskeletal proteins. As shown in Table I, this synthetic peptide binds specifically to intact ankyrin and the ARD, but not the SBD of ankyrin or other cytoskeletal proteins such as spectrin. Control peptides, containing the scrambled sequence (GRATLEGSDKV) with the same amino acid composition as that of the synthetic peptide or another peptide (GTIKRAPFLGP) from a different region (i.e., the sequence between amino acids 399 and 409) of Tiam1, fail to bind any cytoskeletal proteins tested (Table I).

We have also used the synthetic peptide corresponding to Tiam1's amino acid 717–727 sequence to compete for the binding of purified Tiam1 to ankyrin. As shown in Fig. 7 A (c), the synthetic peptide competes effectively with Tiam1 to bind ankyrin with an apparent inhibition constant (*K<sub>i</sub>*) ~0.5 nM. However, control peptides such as GRATLEGSDKV (Fig. 7 A, a) or GTIKRAPFLGP (Fig. 7 A, b) do not compete at all with Tiam1 in ankyrin binding. These results suggest that the amino acid 717–727 sequence of Tiam1 is a critical part of the ankyrin-binding domain of Tiam1. Finally, we have constructed an HA-tagged C1199 Tiam1 deletion mutant lacking the ankyrin binding sequence, amino acids 717–727 (designated as C1199 Tiam1Δ717-727; Fig. 7 B, b). The truncated C1199

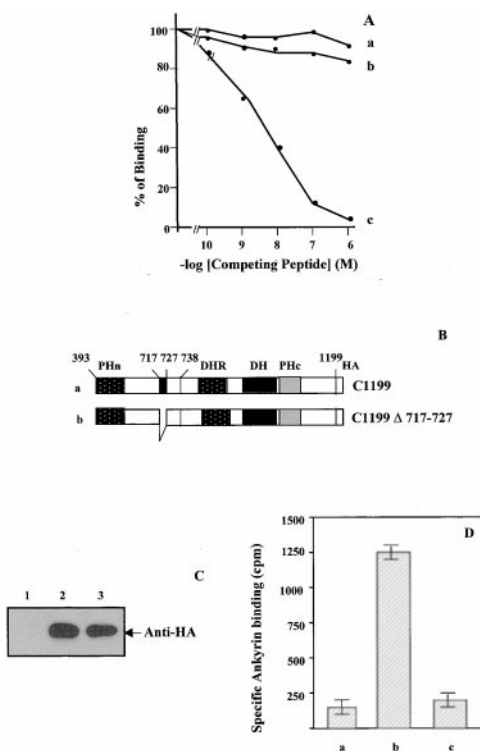
**Table I. Binding of  $^{125}$ I-Labeled Cytoskeletal Proteins to Synthetic Peptides**

	nM $\times$ CPM Bound
<b>Binding to GEGTDAVKRSL</b> (the sequence between amino acids 717 and 727 of Tiam1)	
$^{125}$ I-Labeled ankyrin	15,260 $\pm$ 120
$^{125}$ I-Labeled ARD	14,560 $\pm$ 105
$^{125}$ I-Labeled ankyrin's spectrin binding domain	770 $\pm$ 22
$^{125}$ I-Labeled spectrin	850 $\pm$ 34
<b>Binding to GRATLEGSDKV</b> (the scrambled sequence)	
$^{125}$ I-Labeled ankyrin	1,020 $\pm$ 36
$^{125}$ I-Labeled ARD	920 $\pm$ 29
$^{125}$ I-Labeled ankyrin's spectrin binding domain	901 $\pm$ 24
$^{125}$ I-Labeled spectrin	996 $\pm$ 27
<b>Binding to GTIKRAPFLGP</b> (the sequence between amino acids 399 and 409 of Tiam1)	
$^{125}$ I-Labeled ankyrin	899 $\pm$ 23
$^{125}$ I-Labeled ARD	854 $\pm$ 17
$^{125}$ I-Labeled ankyrin's spectrin binding domain	842 $\pm$ 19
$^{125}$ I-Labeled spectrin	863 $\pm$ 20

$^{125}$ I-labeled cytoskeletal proteins (e.g., intact ankyrin [100 ng] or ARD [100 ng] or spectrin binding domain of ankyrin [100 ng] or spectrin [100 ng] were incubated with nitrocellulose discs coated with either the synthetic peptide GEGTDAVKRSL (corresponding to the sequence between amino acids 717 and 727 of Tiam1), or the scrambled peptide GRATLEGSDKV, or another Tiam1-related peptide, GTIKRAPFLGP (corresponding to the sequence between amino acids 399 and 409 of Tiam1) at 4°C for 4 h as described in Materials and Methods. As a control, the radiolabeled ligands including  $^{125}$ I-labeled ankyrin,  $^{125}$ I-labeled ARD, and  $^{125}$ I-labeled spectrin were also incubated with uncoated beads to determine the binding observed because of the nonspecific binding of various ligands. Nonspecific binding, which represented ~20% of the total binding, was always subtracted from the total binding.

Tiam1 717-727 cDNA (Fig. 7 B, b) and the wild-type C1199 Tiam1 (Fig. 7 B, a) were transiently transfected into SP-1 cells. Our results indicate that both the C1199 Tiam1 $\Delta$ 717-727 mutant (Fig. 7 C, lane 3) and the wild-type C1199 Tiam1 (Fig. 7 C, lane 2) are expressed as a 160-kD polypeptide in SP-1 transfectants using anti-HA-mediated immunoblotting. No protein band was detected in vector-transfected SP-1 cells (Fig. 7 C, lane 1). In vitro binding data reveal that there is a strong binding interaction between ankyrin and HA-tagged C1199 (Fig. 7 D, b). In contrast, the HA-tagged C1199 Tiam1 $\Delta$ 717-727 mutant protein isolated from SP-1 transfectants displays a drastic reduction (~90–95% inhibition) in ankyrin-binding ability (Fig. 7 D, c) compared with the HA-tagged wild-type C1199 Tiam1 (Fig. 7 D, b). No ankyrin binding is observed in materials associated with anti-HA beads using cell lysate isolated from vector-transfected cells (Fig. 7 D, a). These findings suggest that the amino acid 717–727 region is critical for the interaction of Tiam1 with ankyrin.

Most importantly, we have found that the binding of ankyrin (e.g., erythrocyte ankyrin [ANK1], Fig. 8 A, or ANK3's ARD, Fig. 8 B) to Tiam1 significantly increases the GDP/GTP exchange activity of Rac1 GTPase as compared with untreated Tiam1-mediated Rac1 activation (Fig. 8 C). The SBD of ankyrin or other cytoskeletal proteins, such as spectrin, fails to stimulate Tiam1-mediated

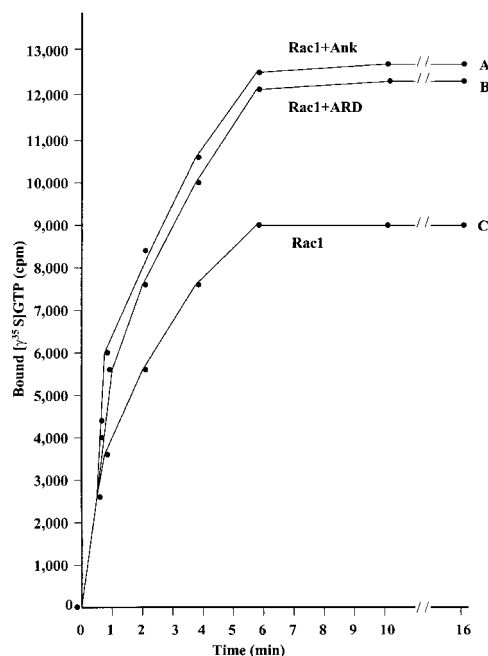


**Figure 7. Identification of the ankyrin binding domain of Tiam1.** (A)  $^{125}$ I-labeled Tiam1 was incubated with ankyrin-coated beads in the presence of various concentrations of unlabeled synthetic peptide (GEGTDAVKRSL, corresponding to the sequence between amino acids 17 and 727 of Tiam1) (c), or the scrambled sequence (GRATLEGSDKV; a), or another Tiam1-related peptide (GTIKRAPFLGP, corresponding to the sequence between amino acids 399 and 409 of Tiam1; b) as described in Materials and Methods. The specific binding observed in the absence of any of the competing peptides is designated as 100%. The results represent an average of duplicate determinations for each concentration of the competing peptide used. (B) Schematic illustration of the in vitro mutagenesis approach used in this study. Both C1199 Tiam1 (a) and C1199 Tiam1 717-727 (lacking the sequence between amino acids 17 and 727; b) were constructed according to the strategy described in Materials and Methods. (C) Anti-HA-mediated immunoblot of SP-1 cells transiently transfected with vector alone (lane 1), or HA-tagged C1199 Tiam1 cDNA (lane 2), or HA-tagged C1199 Tiam1 717-727 cDNA (lane 3). (D) The amount of  $^{125}$ I-ankyrin binding to anti-HA-mediated immunoprecipitates isolated from SP-1 cells transfected with vector alone (a), or HA-tagged C1199 Tiam1 cDNA (b), or HA-tagged C1199 Tiam1 717-727 cDNA (c).

GDP/GTP exchange on Rac1 GTPase (data not shown). Therefore, we believe that ankyrin binding to Tiam 1 plays a pivotal role in the upregulation of Tiam 1-mediated GDP/GTP exchange activity of Rho-like GTPases (e.g., Rac1).

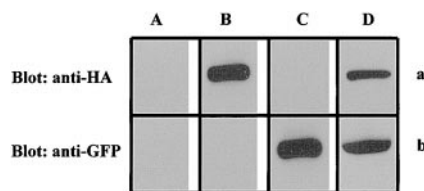
### **Effect of Tiam1 or the Tiam1 Fragment on Rac1 Activation, Tumor Cell Invasion, and Migration**

Previous studies have indicated that both ankyrin and Tiam1 are closely associated with certain tumor-specific behaviors, characterized by an invadopodia structure (or membranous projections) during epithelial tumor cell mi-



**Figure 8.** Stimulation of Tiam1-catalyzed GDP/GTP exchange activity by ankyrin. Purified *E. coli*-derived GST-tagged GTPases (e.g., Rac1, Cdc42, or RhoA) was preloaded with GDP. Subsequently, 2 pmol of Tiam1 (isolated from untransfected or transfected cells according to the procedures described above) was preincubated with no ankyrin or ankyrin (e.g., intact ankyrin or ARD; 1  $\mu$ g/ml), followed by adding to the reaction buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 100  $\mu$ M AMP-PNP, 0.5 mg/ml BSA, and 2.5  $\mu$ M GTP- $\gamma$ -<sup>35</sup>S (~1,250 Ci/mmol). Subsequently, 2.5 pmol of GDP-loaded GST-tagged Rho GTPases (e.g., Rac1, Rac1, or Cdc42) was mixed with the reaction buffer containing Tiam1 and GTP- $\gamma$ -<sup>35</sup>S to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM MgCl<sub>2</sub> as described in Materials and Methods. The termination reactions were filtered immediately through nitrocellulose filters, and the radioactivity associated with the filters was measured by scintillation fluid. The amount of GTP- $\gamma$ -<sup>35</sup>S bound to Tiam1 or control sample (preimmune serum-conjugated Sepharose beads) in the absence of Rho GTPases (e.g., Rac1, Cdc42, or RhoA) was subtracted from the original values. Data represent an average of triplicates from three to five experiments. SD < 5%. (A–C) Kinetics of GTP- $\gamma$ -<sup>35</sup>S bound to GDP-loaded GST-Rac1 by Tiam1 (isolated from SP-1 cells) in the absence (C) or in the presence of ankyrin, e.g., intact erythrocyte ankyrin (ANK1; A) or ARD fragment (B).

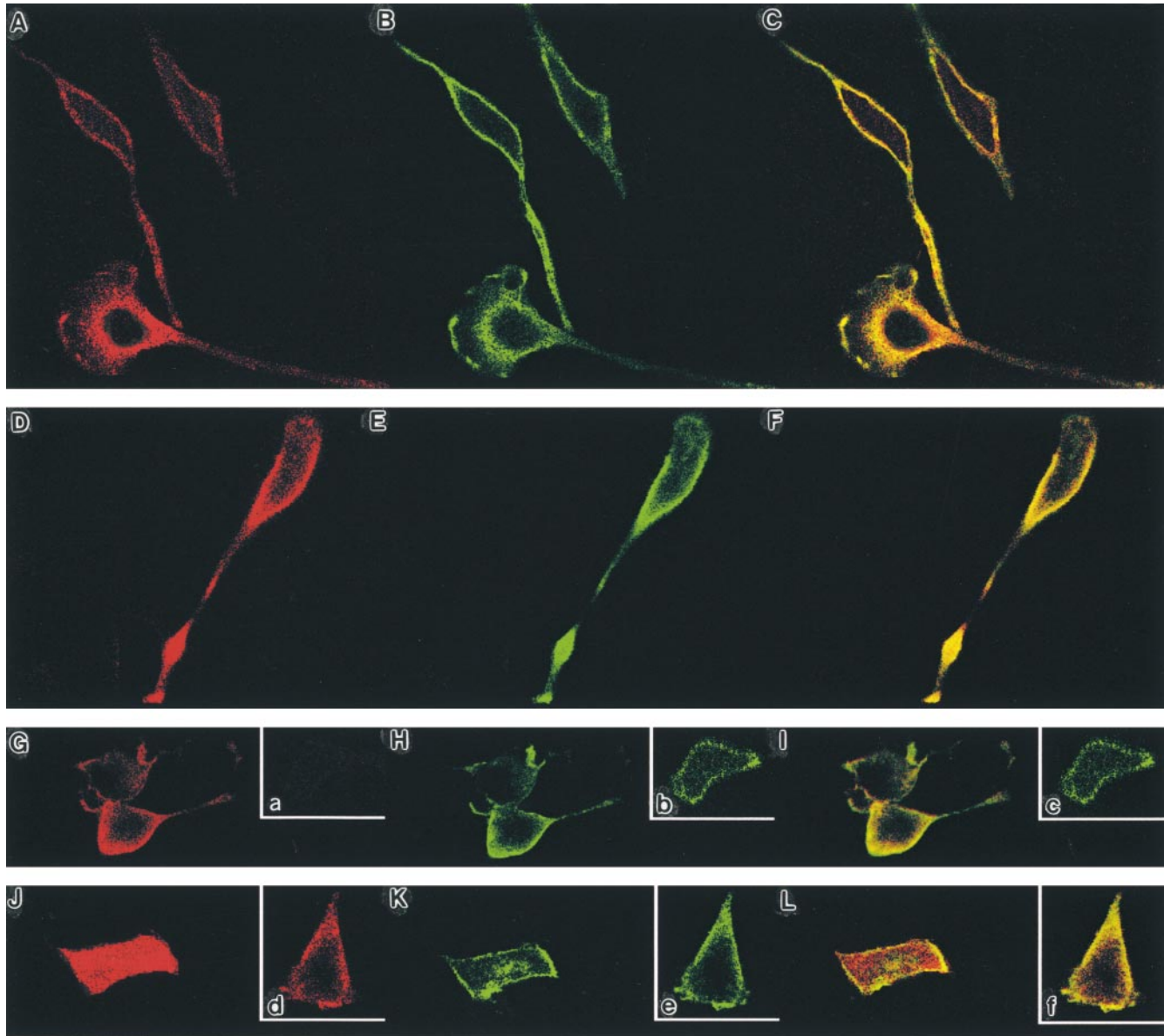
gration (Bourguignon et al., 1998a,b, 2000; Zhu and Bourguignon, 2000). In this study, using double immunolabeling staining, we have observed that both ankyrin (Fig. 10 A) and Tiam1 (Fig. 10 B) are colocalized in the plasma membrane and long projections of SP1 cells (Fig. 10 C). Furthermore, we have transiently transfected breast tumor cells (e.g., SP-1 cells) with HA-tagged NH<sub>2</sub> terminally truncated C1199 Tiam1 cDNA. Our results show that the C1199 Tiam1 is expressed as a 160-kD protein (Fig. 9 B, a) detected by anti-HA-mediated immunoblot in SP1 cells. No protein band was detected in vector-transfected SP1 cells by anti-HA-mediated immunoblotting (Fig. 9 A, a).



**Figure 9.** Transfection of SP1 cells with HA-tagged C1199 Tiam1 cDNA (A) or GFP-tagged Tiam1 fragment cDNA (B) or cotransfection of HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA (C). Detection of C1199 Tiam1 expression by anti-HA-mediated immunoblot in HA-tagged C1199 Tiam1 cDNA-transfected cells (B, a) or in vector-transfected cells (A, a). Detection of Tiam1 fragment expression by anti-GFP-mediated immunoblot in GFP-tagged Tiam1 fragment cDNA-transfected cells (C, b) or vector-transfected cells (A, b). Detection of coexpression of C1199 Tiam1 and Tiam1 fragment by immunoblotting of cells (cotransfected with HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA) with anti-HA antibody (D, a) and anti-GFP antibody (D, b), respectively. In controls, no signal was detected in HA-tagged C1199 Tiam1 cDNA-transfected cells or GFP-tagged Tiam1 fragment cDNA-transfected cells using anti-GFP (B, b) or anti-HA (C, a)-mediated immunoblotting, respectively.

Double immunofluorescence staining data show that ankyrin (Fig. 10 D) and C1199 Tiam1 (Fig. 10 E) are also colocalized on the plasma membrane-related long projections of these C1199 Tiam1 cDNA-transfected cells (Fig. 10 F). Furthermore, we have demonstrated that transfection of SP1 cells with C1199 Tiam1 cDNA stimulates ankyrin-associated Tiam1-catalyzed GDP/GTP exchange on Rac1 (Fig. 11 a), and induces a significant amount of increase in breast tumor cell invasion (Table II, A) and migration (Table II, B) as compared with vector-transfected SP1 transfectants (Fig. 11 b and Table II, A and B). These results are consistent with previous findings indicating that transfection of NIH3T3 cells with the NH<sub>2</sub> terminally truncated C1199 Tiam1 cDNA confers potent oncogenic properties (Van Leeuwen et al., 1995).

Treatment of SP1 cells (e.g., untransfected or transfected cells) with certain agents (e.g., cytochalasin D, a microfilament inhibitor) causes a remarkable inhibition of tumor cell invasion (Table II A) and migration (Table II B). Tiam1-Rac1 signaling initiates oncogenic cascades including c-Jun kinase (JNK) activation, which triggers gene transcription through c-jun and promotes cell transformation (Michiels et al., 1995, 1997). In addition, Tiam1-activated Rac1 stimulates the novel family of serine/threonine kinases, p-21 activated kinases (Manser et al., 1994; Knaus et al., 1995; Bagrodia and Cerione, 1999), which mediates actin assembly and induce the formation of membrane ruffling and lamellipodia (membrane projections). In fact, cytoskeleton-associated membrane projections are often tightly linked to matrix degrading enzymes during breast tumor cell invasion and migration (Bourguignon et al., 1998b). These findings suggest that Tiam1-Rac1 signaling and selective effector(s) play an important role in promoting certain gene expression required for cellular transformation and the upregulation of cytoskeletal changes needed for tumor cell invasion and migration. Identifica-

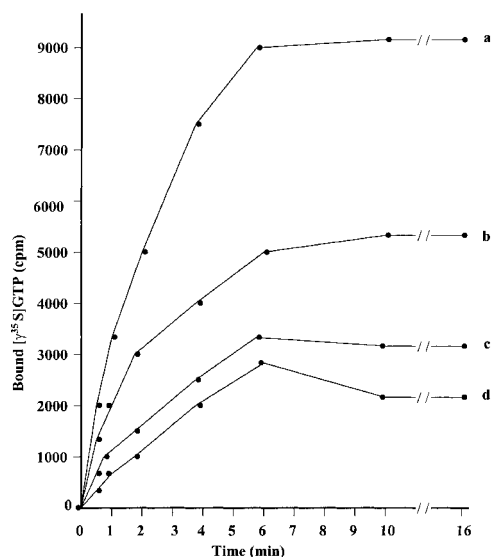


**Figure 10.** Double immunofluorescence staining of ankyrin and Tiam1 in untransfected SP1 cells or SP1 transfectants. SP1 cells (untransfected or transfected with HA-tagged C1199 Tiam1 cDNA or GFP-tagged Tiam1 fragment cDNA or cotransfected with HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA) were fixed by 2% paraformaldehyde. Subsequently, cells were rendered permeable by ethanol treatment and stained with various immunoreagents as described in Materials and Methods. (A–C) Rh-labeled anti-ANK3 staining (A) FITC-anti-Tiam1 staining (B) and colocalization of ankyrin and Tiam1 (C) in untransfected SP1 cells. (D–F) Rh-labeled anti-ANK3 staining (D) FITC-anti-HA-labeled C1199 Tiam1 staining (E), and colocalization of ankyrin and C1199 Tiam1 (F) in HA-tagged C1199 Tiam1 cDNA-transfected SP1 cells. (G–I) Rh-labeled anti-ANK3 staining (G), GFP-tagged Tiam1 fragment (H), and colocalization of ankyrin and Tiam1 fragment (I) in GFP-tagged Tiam1 fragment cDNA-transfected SP1 cells. (a–c) Rh-labeled normal mouse IgG staining (a), GFP-tagged Tiam1 fragment (b), and colocalization of normal mouse IgG and Tiam1 fragment (c) in GFP-tagged Tiam1 fragment cDNA-transfected SP1 cells. (J–L) Rh-labeled anti-HA staining of C1199 Tiam1 (J), GFP-tagged Tiam1 fragment (K), and colocalization of C1199 and Tiam1 fragment (L) in SP1 cells cotransfected with HA-tagged C1199 cDNA and GFP-tagged Tiam1 fragment cDNA. (d–f) Rh-labeled anti-ANK3 staining (d), GFP-tagged Tiam1 fragment (e), and colocalization of ankyrin and Tiam1 fragment (f) in SP1 cells cotransfected with HA-tagged C1199 cDNA and GFP-tagged Tiam1 fragment cDNA.

tion of immediate downstream targets for ankyrin-mediated Tiam1-Rac1 signaling is currently under investigation in our laboratory.

We have also found that SP1 cells transfected with GFP-tagged Tiam1 fragment cDNA express a 68-kD protein as detected by anti-GFP antibody (Fig. 6 B, lane 2; Fig. 9 C, b). In vector-transfected SP1 cells, we are not able to de-

tect any protein band by anti-GFP-mediated immunoblotting (Fig. 9 A, b). Double immunofluorescence staining shows that both ankyrin (Fig. 10 G) and the GFP-tagged Tiam1 fragment (Fig. 10 H) are colocalized in the cell membranes in SP1 transfectants (Fig. 10 I). We believe that the ankyrin staining detected in these SP1 transfectants, revealed by antiankyrin-mediated immunostaining,



**Figure 11.** Kinetics of GTP- $\gamma$ - $^{35}\text{S}$  bound to GDP-loaded GST-Rac1 in the presence of ankyrin-associated Tiam1 isolated from SP-1 cells: transfected with HA-tagged C1199 Tiam1 cDNA (a) or GFP-tagged Tiam1 fragment cDNA (d); or cotransfected with HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA (c) or vector alone (b). Purified *E. coli*-derived GST-tagged GTPases (e.g., Rac1, Cdc42, or RhoA) were preloaded with GDP. First, 2 pmol ankyrin-associated Tiam1 isolated from various SP1 transfectants was added to the reaction buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  AMP-PNP, 0.5 mg/ml BSA, and 2.5  $\mu\text{M}$  GTP- $\gamma$ - $^{35}\text{S}$  ( $\sim 1,250$ , Ci/mmol). Subsequently, 2.5 pmol GDP-loaded GST-tagged Rho GTPases (e.g., Rac1, RhoA, Cdc42 or GST alone) were mixed with the reaction buffer containing ankyrin-associated Tiam1 and GTP- $\gamma$ - $^{35}\text{S}$  to initiate the exchange reaction at room temperature. At various time points, the reaction of each sample was terminated by adding ice-cold termination buffer as described in Materials and Methods. The termination reactions were filtered immediately through nitrocellulose filters, and the radioactivity associated with the filters were measured by scintillation fluid. The amount of GTP- $\gamma$ - $^{35}\text{S}$  bound to Tiam1 or control sample (preimmune serum-conjugated Sepharose beads) in the absence of Rho GTPases (e.g., Rac1, Cdc42, or RhoA) was subtracted from the original values. Data represent an average of triplicates from three to five experiments. SD < 5%.

is specific since no label (Fig. 10 a) is detected in these GFP-Tiam1 fragment-overexpressed cells (Fig. 10 b) using normal mouse IgG (Fig. 10 a). No colocalization (Fig. 10 c) of normal mouse IgG (Fig. 10 a) and GFP-Tiam1 fragment (Fig. 10 b) is observed in these transfectants. Moreover, we have demonstrated that overexpression of the GFP-tagged Tiam1 fragment in SP1 transfectants downregulates ankyrin-associated Tiam1-Rac1 signaling (Fig. 10 d), tumor cell invasion (Table II, A), as well as cell migration (Table II, B).

Finally, cotransfection of SP1 cells with HA-tagged C1199 Tiam1 cDNA and GFP-tagged Tiam1 fragment cDNA was carried out. Using anti-HA or anti GFP-mediated immunoblotting technique, we have detected coexpression of both C1199 Tiam1 (Fig. 9 D, a) and Tiam1 fragment (Fig. 9 D, b) in SP1 transfectants. In controls, no signal was detected in HA-tagged C1199 Tiam1 cDNA-transfected cells or GFP-tagged Tiam1 fragment cDNA-

**Table II.** Measurement of Tumor Cell Invasion and Migration

Cells	Cell invasion* Percent control†	
	DMSO-treated	Cytochalasin D-treated
<b>In vitro cell invasion</b>		
Untransfected cells (control)	100	22
Vector-transfected cells	96	24
Tiam1 fragment cDNA-transfected cell	95	20
C1199 Tiam1 cDNA-transfected cells	155	50
C1199 Tiam1 cDNA and Tiam1 fragment cDNA-cotransfected cells	90	17
<b>In vitro cell migration</b>		
Untransfected cells (control)	100	20
Vector-transfected cells	98	23
Tiam1 fragment cDNA-transfected cell	93	22
C1199 Tiam1 cDNA-transfected cells	158	55
C1199 Tiam1 cDNA and Tiam1 fragment cDNA-cotransfected cells	88	14

\*SP1 cells ( $\sim 10^4$  cells/well in PBS, pH 7.2), in the presence or absence of 20  $\mu\text{g}/\text{ml}$  cytochalasin D (dissolved in DMSO) or DMSO alone, were placed in the upper chamber of the transwell unit. In some cases, SP1 cells were transfected with either HA-tagged C1199 Tiam1 cDNA or GFP-tagged Tiam1 fragment cDNA or HA-tagged C1199 Tiam1 cDNA plus GFP-tagged Tiam1 fragment cDNA, or vector alone. After an 18-h incubation at 37°C in a humidified 95% air/5%  $\text{CO}_2$  atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters containing serum by standard cell number counting assays as described in Materials and Methods. Each assay was set up in triplicate and repeated at least three times. All data were analyzed statistically by *t* test and statistical significance was set at  $P < 0.01$ . In these experiments  $\sim 30$ –40% of input cells ( $\sim 10^4$  cells/well) undergo in vitro cell invasion and migration in the control samples.

†The values expressed in this table represent an average of triplicate determinations of three to five experiments with an SD less than  $\pm 5\%$ .

transfected cells using anti-GFP (Fig. 9 B, b) or anti-HA (Fig. 9 C, a)-mediated immunoblotting, respectively. Furthermore, immunocytochemical staining results show that ankyrin (Fig. 10 d) and the GFP-tagged Tiam1 fragment (Fig. 10 e) are colocalized (Fig. 10 f) in the plasma membranes of SP1 transfectants. In contrast, C1199 Tiam1 (Fig. 10 J) fails to display plasma membrane localization. Consequently, the level of colocalization (Fig. 10 L) between C1199 Tiam1 (Fig. 10 J) and Tiam1 fragment (Fig. 10 K) is greatly reduced. In addition, it is noted that no significant stimulation of long membrane projections was observed in these transfectants (Fig. 10, J–L and d–f). Other tumor-specific behaviors such as Tiam1-Rac1 activation (Fig. 11 c) and cytoskeleton-mediated breast tumor cell invasion (Table II, A) and migration (Table II B) are also greatly inhibited. These findings suggest that the ankyrin-binding domain-containing Tiam1 fragment acts as a dominant negative mutant that effectively competes for ankyrin binding to C1199 Tiam1 in vivo and blocks ankyrin-regulated Tiam1 function associated with tumor-specific phenotypes.

## Discussion

The invasive phenotype of breast tumors, determined by

characteristics such as tumor cell motility and membrane perturbations, is clearly linked to cytoskeletal function. For example, recent studies have shown that certain metastasis-specific molecules (e.g., CD44<sub>v3,8-10</sub> isoform [Bourguignon et al., 1998b, 1999] and its associated matrix metalloproteinase, MMP-9 [Bourguignon et al., 1998b; Yu and Stamenkovic, 1999], as well as Rho kinase [Bourguignon et al., 1999]) are closely associated with the cytoskeleton during tumor cell function. To further examine the regulatory mechanism(s) involved in cytoskeleton-mediated oncogenic signaling leading to tumor cell invasion and migration, we have focused on GEFs (the Dbl or DH family), such as Tiam1, which are known to display oncogenic capability and function as upstream activators of Rho-like GTPases (e.g., Rac1 or Cdc42; Woods et al., 1991; Habets et al., 1994; Michiels et al., 1995; Nobes and Hall, 1995; Van Leeuwen et al., 1995). In breast tumor cells, such as SP-1 cells, Tiam1 is detected as a 200-kD protein (Fig. 1), which is similar to the Tiam1 described in other cell types (Woods and Bryant, 1991; Michiels et al., 1995; Nobes and Hall, 1995; Van Leeuwen et al., 1995, 1997; Hordijk et al., 1997; Stam et al., 1997; Bourguignon et al., 2000). Tiam1, isolated from SP-1 cells, is also capable of carrying out GDP/GTP exchange for Rac1 in vitro (Fig. 2). Sequence analysis of Tiam1 suggests that its association with the invasive and metastatic phenotype is mediated via membrane-linked cytoskeletal regulation and/or activation of Rho family GTPases (Habets et al., 1994; Nobes and Hall, 1995).

Rac1 acts downstream of Tiam1 signaling and regulates the function of several cell adhesion molecules such as the laminin receptor,  $\alpha\beta 1$  integrin (Van Leeuwen et al., 1997), E-cadherin (Hordijk et al., 1997), and the hyaluronan receptor, CD44 (Bourguignon et al., 2000). Tiam1-Rac1 activation also has been shown to be stimulated by certain serum-derived growth activators (e.g., S1P and LPA) during T-lymphoma cell invasion (Stam et al., 1998). However, in epithelial MDCK cells, Tiam1-Rac1 signaling plays an invasion/suppressor role in Ras-transformed MDCK cells (Hordijk et al., 1997). Apparently, various responses by Tiam1-catalyzed Rac1 signaling may be controlled by selective upstream activators (e.g., availability of certain cytoskeletal proteins [e.g., ankyrin], cell adhesion receptors [e.g., CD44, integrin or E-cadherin], growth activators [e.g., serum, S1P, or LPA] or extracellular matrix components [hyaluronic acid, collagen, or fibronectin, etc.]). Moreover, Tiam1 is found to be involved in promoting both Rac1- and RhoA-mediated pathways during neurite formation in nerve cells (Van Leeuwen et al., 1997). The balance between Rac1 and RhoA determines a particular cellular morphology and migratory behavior (Sander et al., 1999).

Ankyrin is a family of membrane-associated cytoskeletal proteins expressed in a variety of biological systems including epithelial cells and tissues (Peters and Lux, 1993). Presently, at least three ankyrin genes have been identified: *ankyrin 1* (Ank 1 or *ankyrin R*), *ankyrin 2* (Ank 2 or *ankyrin B*), and *ankyrin 3* (Ank 3 or *ankyrin G*; Lux et al., 1990; Otto et al., 1991; Kordeli et al., 1995; Peters et al., 1995). These molecules belong to a family of related genes that probably arose by duplication and divergence of a common ancestral gene. Ankyrin is known to bind to a

number of plasma membrane-associated proteins including the following: band 3, two other members of the anion exchange gene family (Bennet, 1992), Na<sup>+</sup>/K<sup>+</sup>-ATPase (Nelson and Veshnock, 1987; Zhang et al., 1998), the amiloride-sensitive Na<sup>+</sup> channel (Smith et al., 1991), the voltage-dependent Na<sup>+</sup> channel (Kordeli et al., 1995), Ca<sup>2+</sup> channels (Bourguignon et al., 1993b, 1995a; Bourguignon and Jin, 1995) and the adhesion molecule CD44 (Bourguignon et al., 1986, 1991, 1992, 1993a; Kalomiris and Bourguignon, 1988, 1989; Lokeshwar and Bourguignon, 1991, 1992; Lokeshwar et al., 1994, 1996). It has been suggested that the binding of ankyrin to certain membrane-associated molecules is necessary for signal transduction, cell adhesion, membrane transport, cell growth, migration, and tumor metastasis (Bennet, 1992; Bourguignon et al., 1995b, 1996, 1997, 1998a; De Matteis and Morrow, 1998; Zhu and Bourguignon, 1998, 2000).

In this study, we have presented new evidence showing the interaction between ankyrin and Tiam1. Specifically, we have demonstrated that Tiam1 and ankyrin (e.g., ANK1 and ANK3) are physically linked to each other as a complex in vivo (Figs. 3 and 10) and in vitro (Figs. 5–7), and that ankyrin binding to Tiam1 promotes Rac1 activation (Figs. 8 and 11). Using purified Tiam1 and GST-tagged ankyrin repeat domain (GST-ARD; Fig. 5) to examine the interaction between Tiam1 and ankyrin in vitro, we have found that ARD is directly involved in the binding of Tiam1 (Fig. 5, C and D, and Fig. 6, C–E). In fact, the binding affinity of ARD to Tiam1 is very comparable to that of intact erythrocyte ankyrin binding to Tiam1 (Fig. 5, C and D). These findings support the conclusion that the ARD fragment of ankyrin is directly involved in the recognition of Tiam1. The 24 ankyrin repeats within the ARD are known to form binding sites for at least seven distinct membrane protein families (Michaely and Bennett, 1995). Often, ARD is organized into four folding subdomains: subdomain 1 (S1), subdomain 2 (S2), subdomain 3 (S3), and subdomain 4 (S4). Recently, we have shown that the S2 subdomain, but not the other subdomains, of ARD binds to the adhesion molecule CD44 directly (Zhu and Bourguignon, 2000). Overexpression of subdomain (S2) of ARD promotes CD44-mediated tumor cell migration (Zhu and Bourguignon, 2000). The question of which ARD subdomain fragment(s) is (are) involved in regulating Tiam1 function remains to be determined.

The structural homology between the ankyrin binding domain of Tiam1 (the sequence between amino acids 717 and 727) and CD44 is quite striking (Lokeshwar et al., 1994). The cytoplasmic domain of CD44 (~70 amino acids long) is highly conserved ( $\geq 90\%$ ) in most of the CD44 isoforms; and it is clearly involved in specific ankyrin binding (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). The ankyrin-binding domain of CD44 has also been mapped using deletion mutation analyses and mammalian expression systems (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998). In particular, the ankyrin-binding domain (e.g., NGGNGTVEDRKPSSEL between amino acids 306 and 320 in the mouse CD44 [Lokeshwar et al., 1994] and NSGNGAVEDRKPSGL amino acids 304 and 318 in human CD44 [Zhu and Bourguignon, 1998]) is required for cell adhesion (Lokeshwar et al., 1994; Zhu and Bourguignon, 1998), the recruitment of Src kinase (Zhu and



Bourguignon, 1998), and the onset of tumor cell transformation (Bourguignon et al., 1998b; Zhu and Bourguignon, 1998). The facts that (1) the recombinant C1199 Tiam1 interacts with ARD fusion protein directly with a stoichiometry of 1:1 (Fig. 6, D and E); (2) a peptide with the sequence <sup>717</sup>GEGTDAVKRS<sup>727</sup>L of Tiam1 binds to ankyrin and ARD but not ankyrin's spectrin binding domain or spectrin (Table I); (3) a Tiam1 peptide (amino acids 717–727) competes with Tiam1 for the binding to ankyrin (Fig. 7 A); (4) the Tiam1 deletion mutant protein (e.g., C1199 Tiam1Δ717–727; Fig. 7 B) fails to bind ankyrin (Fig. 7 D); and (5) ankyrin stimulates Tiam1-catalyzed GDP/GTP exchange activity on Rac1 (Fig. 8) strongly suggest that the sequence (<sup>717</sup>GEGTDAVKRS<sup>727</sup>L) of Tiam1 is an important region for ankyrin binding.

Furthermore, we have shown that transfection of SP-1 cells with HA-tagged NH<sub>2</sub> terminally truncated C1199 Tiam1 cDNA stimulates ankyrin-associated GDP/GTP exchange on Rac1 (Fig. 11) as well as tumor cell invasion (Table II, A) and migration (Table II, B). These Tiam1-activated oncogenic responses are consistent with previous studies indicating that Tiam1-activated Rho-like GTPases may act as downstream effectors of Ras in both tumorigenesis and progression to metastatic diseases (Habets et al., 1994, 1995; van Leeuwen et al., 1995). The amino acids 393–738 Tiam1 fragment (Fig. 6 C) contains not only the putative ankyrin-binding domain (amino acids 717–727), but also the NH<sub>2</sub>-terminal pleckstrin homology (PHn), the coiled-coil region (CC) and an additional adjacent region (Ex) (also designated as PHn-CC-Ex domain; Michiels et al., 1997). This Tiam1 fragment has been shown to be responsible for Tiam1's membrane localization, Rac1-dependent membrane ruffling, and C-Jun NH<sub>2</sub>-terminal kinase activation in fibroblasts and COS cells (Michiels et al., 1997; Stam et al., 1997). In this study, we have found that cotransfection of SP1 cells with Tiam1 fragment cDNA and C1199 Tiam1 cDNA effectively blocks tumor cell-specific behaviors (e.g., C1199 Tiam1 association with ankyrin in the cell membrane [Fig. 10], Rac1 activation [Fig. 11], tumor cell invasion [Table II, A], and migration [Table II B]). These findings further support our conclusion that the ankyrin-binding domain-containing Tiam1 fragment acts as a potent competitive inhibitor, which is capable of interfering with C1199 Tiam1–ankyrin interaction in vivo. Recently, we have also demonstrated that the Tiam1 fragment is required for CD44 (the hyaluronan receptor) binding (Bourguignon et al., 2000). Most importantly, Tiam1–CD44 interaction promotes Rac1 activation and hyaluronic acid-mediated breast tumor cell migration (Bourguignon et al., 2000). These observations clearly suggest that the amino acids 393–738 of Tiam1 contains multiple functional domains (e.g., membrane localization site(s) and cytoskeleton binding domains) required for the regulation of Tiam1–Rac1 signaling and cytoskeleton function. Taken together, we believe that ankyrin–Tiam1 interaction plays a pivotal role in regulating Rac1-activated oncogenic signaling and cytoskeleton-mediated metastatic breast tumor cell progression.

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